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13. ABSTRACT (Maximum 200 words)

This project aims to characterize a novel transcriptional knock out polypeptide (TKO) which we identified in breast cancer cells and which competitively interrupts molecular events in the ISGF3 signaling pathway for interferons-α/β (IFNα). The TKO action is of clinical significance since IFNs-α/β are antiproliferative cytokines with significant potential for therapy of stage II or disseminated breast cancer. Improved chromatographic purification of TKO has been achieved during the initial phase of this project and a single polypeptide of ca. 20 kDa associated with ISGF3 DNA-binding inhibition resolved by two dimensional gel electrophoresis. Biochemical sequence analysis and accumulation of purified TKO product for antibody production is progressing. A second objective of this project has been to explore the interaction of the IFN-α/β signal cascade and the growth stimulatory signal cascade of prolactin (PRL). These molecular cascades can intersect at the level of transductional protein (STAT1, STAT2 of STAT5) phosphorylation. In pilot testing of three cell lines from human breast carcinomas, differences in STAT1 phosphorylation by PRL and IFN-α correlated inversely to the level of original tumor differentiation: least effects of PRL and maximum effects of IFN-α occurred in a cell line negative for estrogen receptors or other differentiation markers.

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(5) INTRODUCTION: Treatment decisions for patients with disseminated breast cancer often pose a difficult problem for oncologists due to the morbidity associated with conventional cytotoxic agents. Many of the conventional cytotoxic agents, such as adriamycin, are mutagenic and increase the risk for subsequent development of leukemia (1). Cytotoxic chemotherapy for stage II breast cancer can be particularly onerous when the cancer is negative for estrogen receptors and the patient is under age 50 years. Interferons (IFNs) are natural cytokines with antiproliferative properties. IFNs-α/β can prevent entry of cancer cells into S-phase of the cell-division cycle (2) and act to cause cell differentiation (3-5). Potential benefits of antiproliferative IFN therapy include the avoidance of damage to normal non-proliferating tissues, mitigation of side-effects upon normal proliferating tissues and elimination of the hazard of delayed carcinogenicity. IFN-α has been tested repeatedly in preclinical models (6-8) and phase I/II clinical trials (7-9) and is including adjuvant therapy in some protocols (10, 11).

We have obtained original evidence that the signal transduction pathway for IFN-α can be selectively interrupted in malignant cells by a negative regulator of transcriptional activation (12). This negative regulator was tentatively identified as a polypeptide with a molecular weight of ca. 20 kDa and was designated as *transcriptional knock out* factor or *TKO* (12). The negative regulatory mechanism of TKO action has been explored and related to inhibition of DNA binding by an auxiliary subunit (48 kDa polypeptide) integral to the IFN-α transcriptional complex (12, 13): the holocomplex (ISGF3) is comprised of two major subunits including ISGF3α (STAT1 and STAT2) and ISGF3γ(48 kDa polypeptide). The auxiliary 48 kDa subunit is common to a family of IFN regulatory factors (IRF) which include a DNA-binding tumor suppressor protein IRF1 and an oncoprotein IRF2 (14).

TKO activity is detected in cell extracts by using electrophoretic mobility gel shift assays (EMSA) with specific oligonucleotide probes representing a highly conserved enhancer region of IFN-stimulated early genes. This region is the *interferon-sensitive response element* (*ISRE*). In preliminary tests, significant TKO activity was identified in two lines of human cells originating from malignant breast lesions. One important purpose of the present work has been to develop the technical capacity to screen patient samples for TKO activity. Identification of TKO in human breast cancers is a potential therapeutic variable and prognostic indicator. While TKO activity can be tested directly in EMSA assays of fresh tissue samples, this approach is practically constrained by sample size, gross uncertainties in sampling of early cancerous lesions and diagnostic imperatives restricting the volume of appropriate tissue available for destructive analysis. A more useful approach will be to develop specific antibodies to the TKO polypeptide, so that properly identified malignant cells in tissue sections can be analyzed for TKO content. Antibody development is a major technical objective of the current project and is being pursued with chromatographic and two dimensional electrophoretic purification of the TKO polypeptide. Twin goals are protein sequence analysis and animal injections of highly purified natural material or appropriate oligopeptides to obtain monospecific or polyclonal TKO antibodies. It is anticipated that the latter will be suitable for studies of frozen biopsy sections, deparaffinized samples of embedded surgical biopsies, or aspiration cytology samples. Success of this work is dependent upon obtaining substantial quantities of highly purified TKO polypeptide(s) and our progress in this direction is described below.

In molecular biologic studies of IFN-α and IFN-gamma signal transduction pathways (15, 16), it has been shown that a critical primary event is phosphorylation of one or more of a family of STAT proteins (signal transducers and activators of transcriptions). The STAT phosphorylation is mediated by particular protein tyrosine kinases (JAK or Tyk) which may autophosphorylate following ligand/receptor binding. The use of polypeptide molecules in the JAK/STAT pathway for signal transduction is common to a number of cytokines in the interleukin family (17), and paradoxically to the polypeptide hormone, prolactin (PRL) (18-21). PRL is one of the principal mammotrophic growth promoters and is a known tumor promoter of the rodent mammary gland (22, 23). We have therefore hypothesized that endogenous PRL could be exercising some role in determining the response of breast cancer cells to IFN-α therapy. An independent objective of the present work has been to explore the possible interactions of PRL and IFN-α during signal transduction. Selective interaction of the phosphorylated 91 kDa STAT protein (STAT1) with an auxiliary polypeptide subunit is key to IFN-α signal specificity, and one aim of the current project is to effect signal modulations which may possibly enhance the antiproliferative activity. Thus, the common roles of JAK and STAT signaling elements in the signal transduction pathways of PRL and IFN-α and their biologic impact at the level of malignant cell proliferation are under investigation. This work involves basic molecular biologic analyses using techniques of gel electrophoresis, immunoblotting and detection of changes in JAK/STAT protein phosphorylation under varied conditions of cell exposures. PRL and IFN-α interactions are being quantitated by EMSA with [^{32}P]-oligonucleotide probes representing the ISRE or the gamma response region (GRR) enhancer elements of genes stimulated by IFN-α (ISRE and GRR) or PRL (GRR only). Proliferation status of the cells is being monitored by cell cycle analyses, including flow cytometric analyses and immunoblot analyses of Rb phosphorylation, cyclin E and cyclin-dependent CDK or CDC kinase activities.

(6) BODY:

Progress in TKO purification: Since initiation of the project, the purification of protein with TKO activity has been refined under the direction of Dr. E. Petricoin and samples have been made available for peptide mapping and amino acid sequence analyses in a collaborating laboratory of Dr. Donald Hunt at the University of Virginia Department of Chemistry. In the original work cell extracts were chromatographically separated at 4°C using heparin-sepharose (Pharmacia) with a 0 to 0.3 M NaCl gradient and hydroxylapatite (Bio-Rad) with a 0-0.3 M Na_2PO_4 (pH 7.0) elution gradient (12). These procedures have been substantially improved during the initial phase of this project. Currently, cells are dounce homogenized in Buffer A (20 mM KCL; 10 mM HEPES @ pH7; 5 mM dithiothreitol) with 20% glycerol and 0.1% NP40. Cytoplasmic extract is obtained by centrifugation of the whole cell lysate as ca. 8000 x G (10000 rpm) for 30 min. The supernatant is loaded over a heparin-sepharose resin. The flow-through and two column washes are collected and pooled, then loaded onto a hydroxylapatite column. The active fraction elutes in a 0.2-0.4 M sodium phosphate gradient. It is brought to 35% ammonium sulfate final concentration and spun at 8000 x G for 30 minutes. A detergent layer from the top of the tube is collected. This material is further purified by loading onto a phenyl-Sepharose hydrophobic interaction column equilibrated with 35% ammonium sulfate/Buffer A without detergent. The flow-through material then is collected and

applied to a T-butyl hydrophobic interaction C column at 35% ammonium sulfate. This further eliminates non-specific proteins before final concentration on an hydroxylapatite column. Fig. 1 shows TKO activity in a series of fractions eluted from the hydroxylapatite column used in the final purification step. Specific activity, shown by competitive inhibition of complex formation in the EMSA for interferon-induced ISGF3 was increased $>10^4$ X based upon relative protein concentrations in crude and purified extracts (not shown).

As shown in Fig. 1, C33a human cervical carcinoma cells grown in suspension cultures provide a practical and standard source of TKO protein for large scale purification. The presence of a TKO activity in crude cytoplasmic extracts from certain breast cancer cell lines was indicated in preliminary experiments for this project. Since initiation of this project, we have been able to purify the active TKO fraction from ZR 75-1 breast cancer cells. The latter cells originated from a case of disseminated infiltrating ductal carcinoma with malignant ascites (24). Comparison of TKO activity from ZR 75-1 whole cell extracts and from the C33a cells is shown in Fig. 2. The sample in lane #3 of Fig. 2 represents a maximally purified fraction from ZR 75-1. Electrophoretic mobility of the polypeptide(s) with TKO activity in the ZR-75-1 breast cancer cells and C33a was compared by two dimensional gel electrophoresis (2D-gels) of the most highly purified extracts eluted from the chromatographic matrices as described above. Figs. 3 represents 2D-gels of these highly purified whole cell extracts stained with silver nitrate (3A from C33a, 3B from ZR-75-1). Despite the exhaustive chromatographic purification described above approximately a dozen clearly delineated polypeptide spots were resolved (Fig. 3). In order to distinguish the spot(s) with specific TKO activity, proteins were depleted by ligation to a synthetic N-terminal fragment (125 AA) of ISGF3γ (48 kDa IRF-family polypeptide) conjugated to sepharose-glutathione beads. The ISGF3γ fragment was amplified from cDNA in E.coli PGEX in a frame for the GST fusion protein. It was extracted by lysis and sonication of E.Coli in PBS with PMSF and 0.1% triton-X 100. Beads were loaded for 2 hr at 4°C in a 50% slurry, thoroughly washed X 2 at 8000 x g in PBS-triton-X, and stored on ice. Just before use the loaded beads were washed in ice cold buffer A and mixed as a 50% slurry with TKO-enriched samples of purified whole cell extract that had been eluted from the final hydroxylapatite column and dialyzed against buffer A. After reaction at 4°C overnight, with continuous agitation, the beads were sedimented at 8000 x g and samples of the supernatant were analyzed on to 2D-gels. A spot indicated by the arrows in Fig. 3 was found to be significantly diminished in intensity after silver or Coomassie blue staining (not shown) while other spots were relatively unaffected. Parallel gel shifts showed that the supernatant had lot the competitive inhibitory activity for DNA binding by ISGF3. A resolved product at ca. 20 kDa therefore has tentatively been identified as the TKO and corresponding samples from preparative 2-D gels have been sent to Dr. Donald Hunt at the University of Virginia in Charlottesville for amino acid sequence analyses.

Pilot analyses of interaction of PRL and IFN-α on JAK/STAT signaling in human breast cancer cells. Three PRL-responsive human breast carcinoma lines (T47D, MCF-7 and BT-20) were tested for PRL and IFN-α-induced signaling via STATI and STAT2. Cell dependent effects were noted: in T47D cells PRL and IFN-α induced comparable STAT1 tyrosine phosphorylation responses as determined by phosphotyrosine immunoblotting. The effect of PRL proved additive

to that of IFN-α. In MCF-7 cells PRL, but not IFN-α induced early STAT1 tyrosine phosphorylation. In estrogen-receptor-negative and morphologically less differentiated BT-20 cells, IFN-α induced marked phosphorylation of STAT1 and STAT2, whereas PRL had only a minimal effect on STAT1. Pretreatment of T47D or MCF-7 cells with IFN-gamma for 24 hr served to enhance the PRL effect on phosphorylation of STAT1, possibly due to stimulation of JAK2 phosphorylation.

Fig. 1 TKO activity in human C33a cell extract indicated by competitive inhibition in EMSA of human fibroblast ISGF3 binding to a ^{32}P-end labeled double stranded synthetic oligonucleotide with the sequence 5'GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCC3' which corresponds to the interferon sensitive response element (ISRE) from the ISG15 promoter. In this competitive binding assay, a series of fractions of cytoplasmic extract from whole cell lysate eluted from an hydroxylapatite column with a sodium phosphate gradient were tested and compared to negative control samples which show maximum gel retardation of ^{32}P-oligonucleotide probe by induced ISGF3: sample "HF" (lane #1) was a crude cytoplasmic extract of human fibroblasts after treatment with IFN-α (i.e. positive for ISGF3) and shows gel retardation of the ^{32}P probe (arrow). The sample "C33" (lane #2) is a known inactive fraction of hydroxylapatite eluate from C33a whole cell extract. Sample TKO (lane #3) is a known positive control sample from a frozen aliquot of highly purified TKO to show maximum competitive inhibition of the ISGF3 gel shift. Note a sharp peak of competitive inhibitory activity in the eluate beginning at lane #7 from the right and tailing of the activity in samples of fractions applied to lanes #10 and #11.

Fig. 2 TKO activity in a human breast cancer cell (ZR-75-1) extract indicated by competitive inhibition in an EMSA representing human fibroblast ISGF3 binding to a ^{32}P-end labeled double stranded synthetic oligonucleotide probe corresponding to the ISRE from the ISG15 promoter (same as Fig. 1). The sample "HF" in lane #1 was crude extract from human fibroblasts treated with IFN-α and shows the maximum gel retardation of ^{32}P-oligonucleotide probe by IFN-induced ISGF3. The sample "ZR" in lane #3 is a fraction of sodium phosphate eluate from an hydroxylapatite column with peak competitive inhibitory binding activity in the EMSA assay (similar to lanes #7-9 in Fig. 1) It should be compared to the inhibitory activity evident in sample C33 (lane #2) which comes from a highly purified C33a extract. The sample "HeLa" in lane #4 contains crude extract from human cervical carcinoma cells (HeLa) known to be negative for TKO activity (12), and there is no competition with ISGF3 binding to the ^{32}P-oligonucleotide probe (arrow).

Fig. 3 Examples of TKO resolution in 2-D gels. Positive images represent examples of purified whole cell extracts from C33a (3A) and ZR-75-1 (3B) cells stained with silver nitrate after electrophoresis as described: Immobilized ampholine gradient strips (pH3-10 non-linear) were obtained from Pharmacia and run under conditions outlined by the manufacturer. The strips were rehydrated overnight in a solution of 8M urea, 4% CHAPS, and 75 mM DTT, with bromophenol blue and 1% pharmalytes (pH 3-10). The strips were then placed onto an immobilon horizontal gel box and loaded with sample diluted in a 2X stock of 8 M urea, 4% CHAPS, 75 mM DTT, and 40 mM TRIS. The strips were run under silicon oil at 3500 volts

for 48 hr. The strips were then placed in equilibration buffer consisting of 4%SDS, 0.025 M TRIS pH 6.8, 30% glycerol, 1% iodoacetamide for 10 min, and loaded onto standard Laemelli SDS gels (15%) and run for 3 hrs. The stained spots indicated by arrows were eliminated when chromatographically enriched extract samples were depleted of TKO by incubations with a synthetic fragment of ISGF3γ as discussed in the above text.

(7) CONCLUSIONS: Analyses to date confirm that a negative regulator of transcriptional activation by IFN-α, which was identified in crude extracts of human breast cancer cells, corresponds in electrophoretic mobility to a ca. 20 kDa polypeptide purified from human cervical carcinoma cells and previously designated *TKO* (transcriptional knock-out factor). The correspondence was shown in direct comparisons of whole cell extracts purified by a series of column chromatographic steps and subjected to two-dimensional gel electrophoresis. Depletion tests with a synthetic fragment of ISGF3γ conjugated to sepharose-glutathione beads verified identity of the resolved 20 kDa polypeptide on 2D gels with TKO competitive binding activity. Sequence analysis of the highly purified material is in progress and we are currently accumulating purified material for inoculation of experimental animals. The aim in year 2 to is to generate and pilot test monoclonal or polyclonal antibodies per original statement of work. In a second line of work, as originally proposed, tests of PRL signaling in three PRL-responsive human breast carcinoma lines (T47D, MCF-7 and BT-20) indicated that PRL-dependent STAT1 tyrosine phosphorylation was cell-dependent: different responses were observed in cells from well differentiated, estrogen-receptor-positive malignancies as compared to cells from a poorly differentiated malignancy. These initial findings suggest an inverse correlation of IFN and PRL effects and tentatively support our original hypothesis that changes in cytokine or growth factor signalling may parallel neoplastic evolution and pertain to individualization of patient treatment protocols.

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Competitive inhibition of DNA binding complex formation (EMSA)

Fig - 1

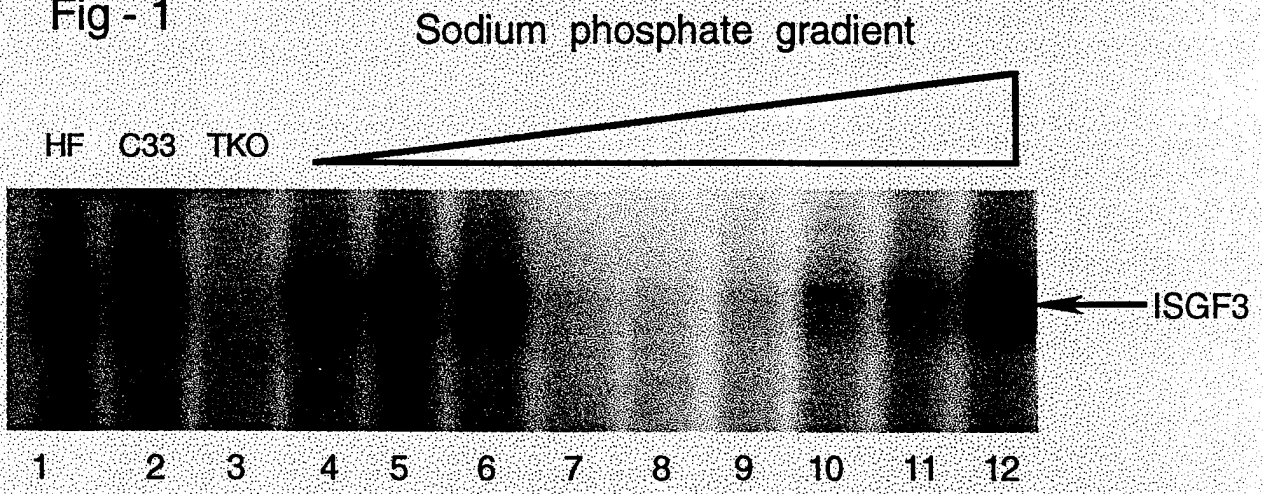
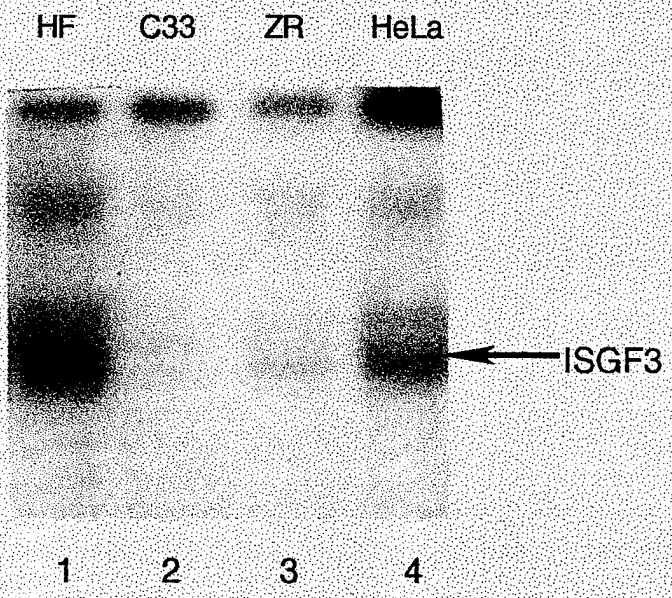


Fig - 2



Purified TKO / 2D gels

Fig - 3A C33a extract

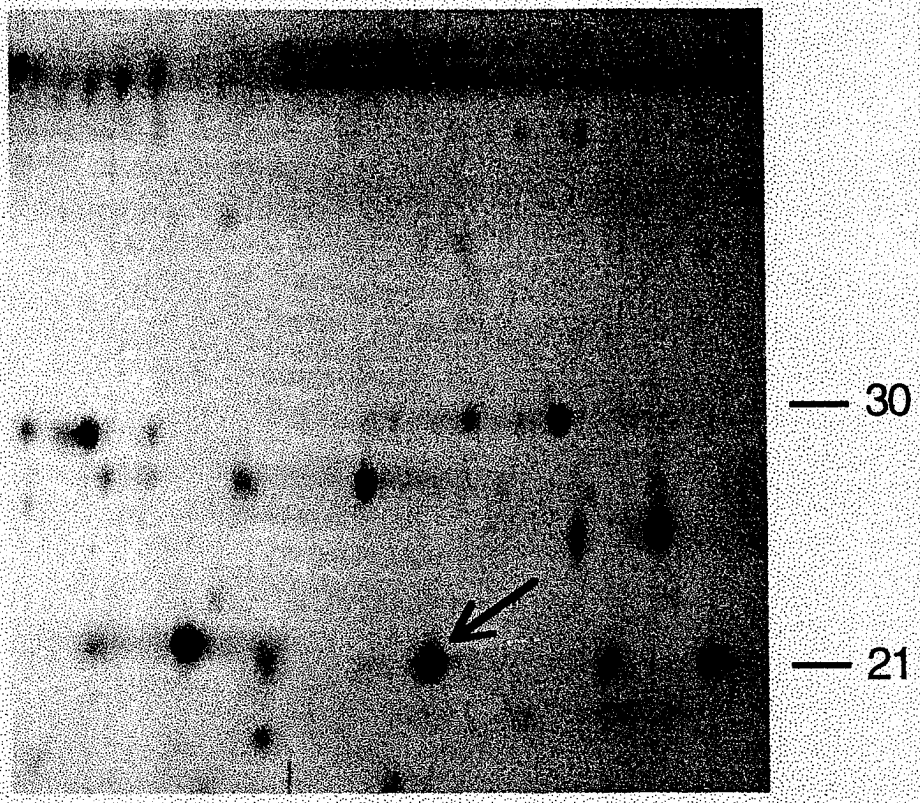
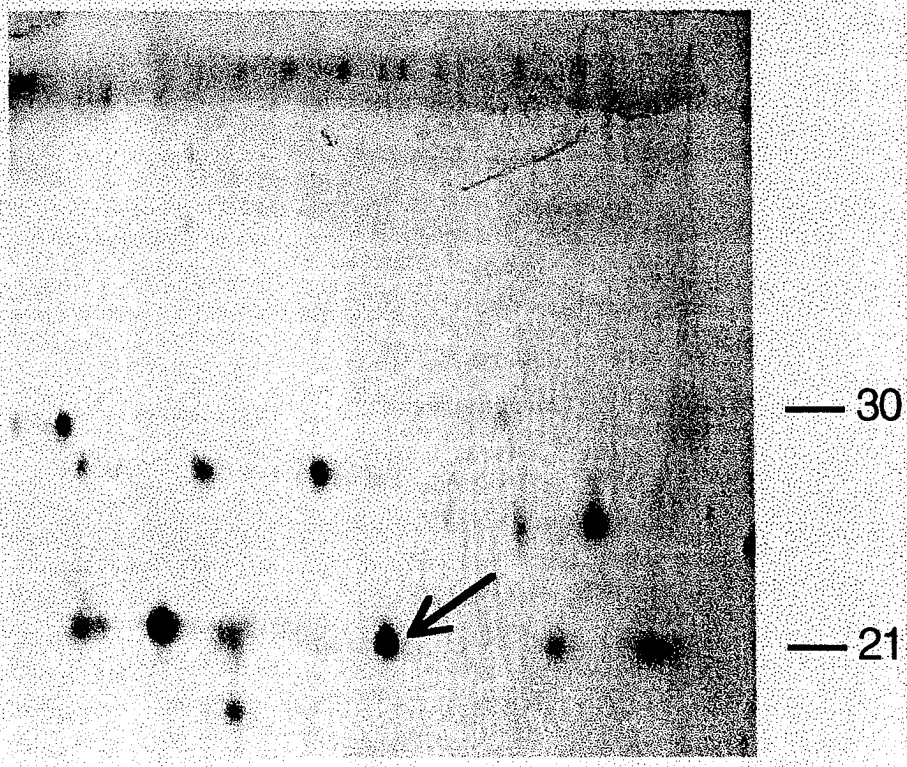



Fig - 3B ZR 75-1 extract